# The Final Step in 5.8S rRNA Processing Is Cytoplasmic in Saccharomyces cerevisiae

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# The Final Step in 5.8S rRNA Processing Is Cytoplasmic in *Saccharomyces cerevisiae*▽†

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**The 18S rRNA component of yeast (*Saccharomyces cerevisiae*) 40S ribosomes undergoes cytoplasmic 3′ cleavage following nuclear export, whereas exported pre-60S subunits were believed to contain only mature 5.8S and 25S rRNAs. However, *in situ* hybridization detected 3′-extended forms of 5.8S rRNA in the cytoplasm, which were lost when Crm1-dependent preribosome export was blocked by treatment with leptomycin B (LMB). LMB treatment rapidly blocked processing of 6S pre-rRNA to 5.8S rRNA, leading to TRAMP-dependent pre-rRNA degradation. The 6S pre-rRNA was coprecipitated with the 60S export adapter Nmd3 and cytoplasmic 60S synthesis factor Lsg1. The longer 5.8S+30 pre-rRNA (a form of 5.8S rRNA 3′ extended by ~30 nucleotides) is processed to 6S by the nuclear exonuclease Rrp6, and nuclear pre-rRNA accumulated in the absence of Rrp6. In contrast, 6S to 5.8S processing requires the cytoplasmic exonuclease Ngl2, and cytoplasmic pre-rRNA accumulated in strains lacking Ngl2. We conclude that nuclear pre-60S particles containing the 6S pre-rRNA bind Nmd3 and Crm1 and are exported to the cytoplasm prior to final maturation by Ngl2.**

Ribosomes are composed of a large, 60S subunit containing three rRNA species—the 25S, 5.8S, and 5S rRNAs—and a small, 40S subunit containing the 18S rRNA species. The 18S, 5.8S, and 25S rRNAs are cotranscribed in the nucleolus as a polycistronic transcript (Fig. 1A) that undergoes a complex series of endonucleolytic cleavages and exonucleolytic processing steps to yield the mature rRNAs (Fig. 1B). Processing of pre-rRNAs occurs within preribosomal particles that contain, in addition to the pre-rRNA and ribosomal proteins, some 200 processing, modification, and assembly factors and 75 small nucleolar RNAs.

The preribosomes that will form the 40S and 60S particles are separated by pre-rRNA cleavage at site $A_2$. This cleavage can occur cotranscriptionally, within the small subunit (SSU) processome (31), or posttranscriptionally in the 90S preribosome (Fig. 1B). In either case, the released pre-40S subunit containing the 20S pre-rRNA is rapidly exported from the nucleus to the cytoplasm, where maturation to 18S rRNA is completed (42). 60S subunit assembly requires more extensive rearrangement and processing within the nucleolar and nucleoplasmic compartments prior to export to the cytoplasm, and multiple different pre-60S particles have been identified via affinity purification (3, 11, 15, 30, 34, 35). During 60S maturation, the 27S pre-rRNAs are 5′ processed to sites $B_{1L}$ and $B_{1S}$, the 5′ ends of the $5.8S_L$ and $5.8S_S$ rRNAs, respectively. Subsequent cleavage at site $C_2$ generates the 26S pre-rRNA, which is 5′ processed to 25S rRNA, and $7S_L$ and $7S_S$, which are 3′ processed to $5.8S_L$ and $5.8S_S$ rRNAs, respectively. Processing of 7S pre-rRNA to 5.8S rRNA is a multistep pathway, requiring the activity of several 3′→5′ exonucleases. 7S pre-rRNAs are initially 3′ processed to 5.8S+30 (forms of $5.8S_L$ and $5.8S_S$ that are 3′ extended by ~30 nucleotides [nt]) by the nuclear exosome complex (1). The 5.8S+30 species are then processed to $6S_L$ and $6S_S$, heterogeneous populations of pre-5.8S species that are 3′ extended by ~5 to 8 nt. Strains lacking the exosome-associated exonuclease Rrp6 strongly accumulate 5.8S+30 (8). However, a majority of pre-rRNA molecules are correctly processed, making it unclear whether Rrp6 is the only major player in normal 3′ processing of 5.8S+30. Processing of 6S pre-rRNA to mature 5.8S appeared to strictly require the activity of the putative nuclease Ngl2 (10) (Fig. 1B). The situation was, however, complicated by the observation that strains lacking combinations of the Rex1, Rex2, and Rex3 exonuclease (44), or components of the exosome (1), also showed 6S pre-rRNA accumulation. Since strains lacking Ngl2 are viable, 60S ribosomes containing the 6S pre-rRNA are functional in protein synthesis (10). The high-resolution cryo-electron microscopy (cryo-EM) structure of the yeast (*Saccharomyces cerevisiae*) ribosome (36) indicates that the 3′ end of the 5.8S rRNA is exposed on the surface of the particle, where it is available for processing and would not be predicted to interfere with ribosome function.

Export of 40S and 60S subunits from the nucleus to the cytoplasm requires active transport through nuclear pore complexes. The small GTPase Ran (Gsp1 and Gsp2 in yeast) provides the directionality for this transport system. In the nucleoplasm, Ran-GTP promotes formation of complexes between export receptors termed "exportins" and export substrates. Following transport, these complexes dissociate in the cytoplasm when GTP is hydrolyzed. The export receptor for both pre-40S and pre-60S subunits in yeast and higher eukaryotes is the exportin Crm1/Xpo1, a member of the karyopherin β family (13, 17, 28, 38, 41). Exportins bind to leucine-rich nuclear export signals (LR-NES) present on either the cargo or on adapter proteins. During 60S export, Crm1/Xpo1 interacts

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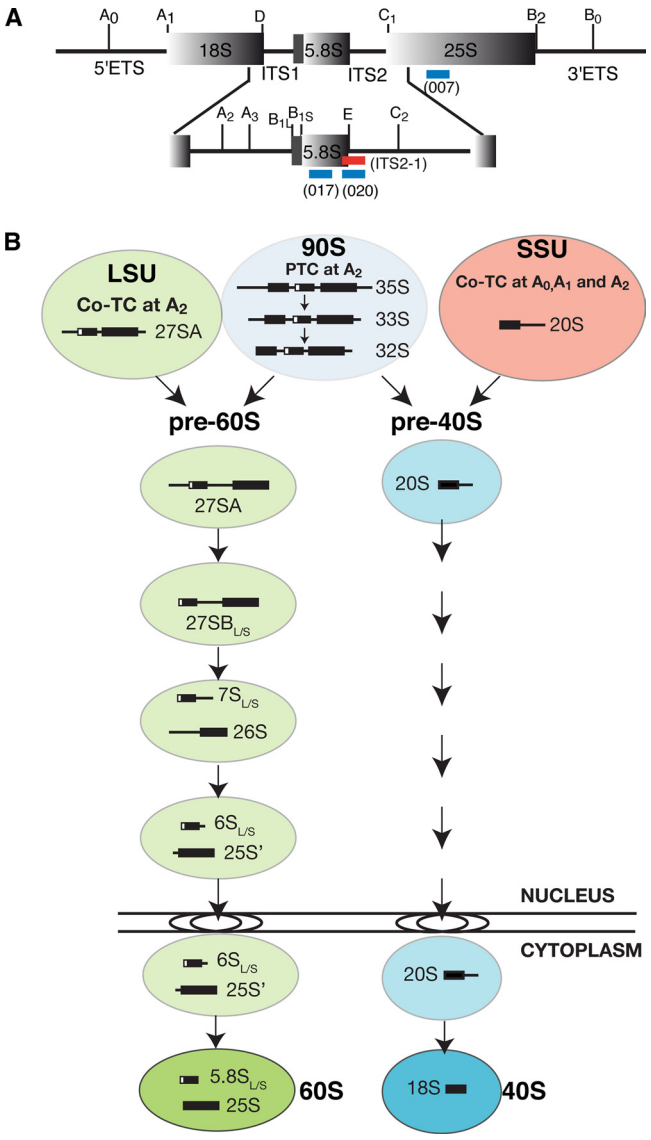



FIG. 1. Pre-rRNA processing pathway in *Saccharomyces cerevisiae*. (A) The structure of the 35S rRNA precursor and locations of processing sites. The pre-rRNA encodes the 18S, 5.8S, and 25S rRNAs, which are flanked by the 5′ and 3′ external transcribed spacers (5′-ETS and 3′-ETS) and separated by internal transcribed spacers 1 and 2 (ITS1 and ITS2). The positions of oligonucleotide probes used for Northern hybridization and FISH analysis are shown in blue and red boxes, respectively. (B) The pre-rRNA processing pathway. RNA polymerase I (Pol I) transcripts undergo one of two alternate fates. The 35S precursor, contained within the 90S preribosome, is generated by cleavage at site $B_0$ within the 3′-ETS. This is followed by posttranscriptional endonucleolytic cleavages (PTC) within the 5′-ETS, at $A_0$ and $A_1$ at the 5′ end of the mature 18S rRNA and within ITS1 at $A_2$. Cleavage at $A_2$ separates the precursors to the 40S and 60S subunits and generates the 20S and $27SA_2$ pre-rRNAs. Alternately, Pol I transcripts can undergo cotranscriptional cleavage (Co-TC) at the $A_0$, $A_1$, and $A_2$ sites, within the small subunit (SSU) processome. Co-TC cleavage is followed by the assembly of the large subunit (LSU) processome on the nascent RNA transcript. Following either posttranscriptional or cotranscriptional cleavage, the 20S pre-rRNA, contained within a pre-40S particle, is exported from the nucleus to the cytoplasm, where maturation to 18S is completed. The pathway of 5.8S and 25S synthesis occurs within a series of pre-60S particles. The $27SA_2$ pre-rRNA follows one of two alternate pathways: around 85% is cleaved at the $A_3$ site within ITS2, followed by 5′→3′ exonucleolytic processing generating the $27SB_s$ pre-rRNA. The remaining 15% is processed at site $B_{1L}$, which is located 8 nt 5′ to $B_{1S}$, yielding the $27SB_L$ pre-rRNA. These two alternate forms of 27SB are cleaved within ITS2 at site $C_2$, yielding 26S pre-rRNA and the long and short forms of 7S. The 7S pre-rRNAs are converted to $6S_L$ and $6S_S$ by the nuclear exosome and Rrp6. Maturation of 26S to 25S rRNA proceeds by a two-step 5′-exonuclease pathway. Subsequently, pre-60S particles are exported to the cytoplasm, where final maturation to 5.8S is completed.

with adapter Nmd3, which is likely to bind the pre-60S particle in the nucleus immediately prior to its export (13, 17). In addition, two export receptors that do not rely directly on the Ran cycle, the heterodimer Mex67/Mtr2 and Arx1, participate in yeast pre-60S export (7, 46).

Early analyses of yeast ribosome synthesis established that exported pre-40S ribosomes contained the 20S pre-rRNA (42), whereas exported pre-60S particles were always believed to contain the fully matured 5.8S and 25S rRNAs. However, apparent links have emerged between pre-60S export and the final step in 5.8S maturation. Yeast mutants defective in components of the export machinery, including the yeast Ran homologue Gsp1, accumulated 3′-extended forms of 5.8S pre-rRNA in the nucleoplasm (37), while loss of late-acting 60S synthesis factors inhibited both 5.8S maturation and export (30). These links may be conserved in evolution, since 3′ processing of 5.8S rRNA in *Xenopus* oocytes is apparently also linked to pre-60S export (41), while the cytoplasmic 3′ exonuclease Eri1 is responsible for the final steps in 5.8S maturation in *Caenorhabditis elegans* (12).

Here we investigate the links between 5.8S formation and pre-60S subunit export and report that a 3′-extended precursor to 5.8S rRNA, rather than mature rRNA, is exported to the cytoplasm.

## MATERIALS AND METHODS

**Strains and microbiological techniques.** Standard procedures were used for the propagation and maintenance of yeast. A full list of strains used in this study can be found in Table 1. YET31 and YET32 were constructed using a one-step PCR strategy (27). Transformants were selected for resistance to nourseothricin (NAT) and screened by PCR and appearance of a cold-sensitive phenotype, conferred by *trf4*Δ. For pulse-labeling and pulse-chase analyses, strains were transformed with a p*URA* vector to confer uracil prototrophy.

**FISH.** For fluorescence *in situ* hybridization (FISH), cells were fixed in 3.7% paraformaldehyde at room temperature, spheroplasted using Zymolyase, and dehydrated in 70% ethanol overnight at −20°C. Locked-nucleic acid (LNA) modified probe (Exiqon) was fluorescently labeled using a ULS Cy3 kit (GE Lifescience). The sequence of the internal transcribed spacer 2-1 (ITS2-1) probe used is TTTGAGAAGGAAATGACGCT, with a predicted melting temperature ($T_m$) of 74°C. Fluorescence *in situ* hybridization was performed as previously described (26), with modifications to hybridization and wash conditions. Hybridization was performed in 80% formamide–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 45°C. Following hybridization, coverslips were washed extensively under the following conditions. Two washes in 80% formamide–2× SSC at 45°C, 1 wash in 2× SSC–0.1% Triton at 25°C, 1 wash in 2× SSC at 25°C, and a final wash in 1× phosphate-buffered saline (PBS). Cells were stained with DAPI (4′,6-diamidino-2-phenylindole) to visualize DNA and coverslips were mounted in Pro-Long mounting medium (Invitrogen). All Images were captured using a Coolsnap charge-coupled device (CCD) camera fitted to the DeltaVision RT Restoration imaging system based on the Olympus IX71 microscope using ×100 objective with an NA of 1.4. Images captured using the DeltaVision system were subjected to real-time two-dimensional deconvolution algorithms. Single optical sections were selected following deconvolution and assembled using Image J software.

TABLE 1. Yeast strains used and constructed in this study

| Strain | Genotype | Reference or source |
|---|---|---|
| BMA38 | *MAT***a** *his3Δ200 leu2-3,112 ura3-1 trp1Δ ade2-1* | 4 |
| BY4741 | *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* | 6 |
| CEN | *MAT***a** *ura3-52 trp1-289 leu2-3,112 his3 D1 MAL2-8C SUC2* | 9 |
| W303 | *MAT***a** *ura3-52 trp1Δ2 leu2-3,112 his3-11 ade2-1 can1-100* | |
| MNY7 | Same as W303, but Δ*CRM1*::Kan$^r$ pDC-*CRM1* (*LEU2*/*CEN*) | 29 |
| MNY8 | Same as W303, but *CRM1*Δ::Kan$^r$ pDC-*CRM1* ($T_{539}C$) (*LEU2*/*CEN*) | 29 |
| YET20 | Same as MNY7, but p*URA* | This study |
| YET21 | Same as MNY8, but p*URA* | This study |
| YET22 | As BY4741, but *ARX1-TAP*::*HIS3 MX6* | 14 |
| YET23 | As BY4741, but *NMD3-TAP*::*HIS3 MX6* | 14 |
| YET24 | As BY4741, but *LSG1-TAP*::*HIS3 MX6* | 14 |
| YET25 | As BY4741, but *CRM1-TAP*::*HIS3 MX6* | 14 |
| YET26 | As BMA38, but p*URA* | This study |
| YCA12 | As BMA38, but *rrp6*Δ::*Kl TRP* | 1 |
| YET27 | As YCA12, but p*URA* | This study |
| YET28 | As BY4741, but p*URA* | This study |
| YET29 | As BY4741, but *ngl2*Δ::*KAN MX6* | 45 |
| YET30 | As YET29, but p*URA* | This study |
| YET31 | As MNY7, but *trf4*Δ::*NAT MX6* | This study |
| YET32 | As MNY8, but *trf4*Δ::*NAT MX6* | This study |

**Pulse-labeling and pulse-chase analysis.** Pulse-labeling was performed as recently described (M. Kos and D. Tollervey, submitted for publication). Briefly, *CRM1* and *crm1*($T_{539}C$) (*crm1* with amino acid alteration $T_{539}C$) strains were pregrown in synthetic medium lacking uracil. Cells with an optical density at 600 nm ($OD_{600}$) of 0.3 to 0.4 were labeled with [8-$^3$H]uracil (1 mCi in 25 ml culture) for 5 min followed by treatment with leptomycin B (LMB; 100 ng ml$^{-1}$). One-milliliter cell samples were fixed in 10 ml of ethanol prechilled to −80°C on dry ice. Cells were then pelleted and washed in water, and RNA was extracted and analyzed as described below. Tritiated RNAs were visualized using a Fuji phosphorimager system and quantified using AIDA software. Pulse-chase analysis was performed in a similar manner, except that following 2 min of uracil labeling, a chase was performed by adding an excess of unlabeled uracil.

**RNA extraction, Northern hybridization, and primer extension.** RNA was extracted as previously described (40) and resolved on standard 8% acrylamide–8.3 M urea gels. Primer extension reactions were carried out as previously described (5). Oligonucleotides for Northern hybridizations and primer extension can be seen in Fig. 1A, and the sequences are as follows: 007, 5′-CTCCG CTTATTGATATGC; 017, 5′-GCGTTGTTCATCGATGC; and 020, 5′-TGAG AAGGAAATGACGCT.

**Affinity purification of TAP-tagged strains.** One-step affinity purification of tandem affinity purification (TAP)-tagged strains was performed as previously described (33) with the following modifications. Exponentially growing yeast cells ($OD_{600}$ of 0.6) were harvested by centrifugation and frozen in liquid nitrogen. Total extract was prepared by adding 1 packed cell volume (PCV) of zirconia beads and 1 PCV of immunoprecipitation (IP) buffer (100 mM KCl, 50 mM HEPES [pH 7.5], 5 mM $MgCl_2$, 0.1% NP-40, 1 mM dithiothreitol [DTT], Complete protease inhibitor tablet) and vortexed for 5 min. Lysed cells were incubated with IgG-Sepharose for 1 h. Beads were extensively washed in IP buffer prior to RNA extraction. Associated pre-RNAs were recovered from the IgG beads or the total extract by guanidinium and phenol-chloroform extraction and ethanol precipitation. RNA was analyzed by Northern hybridization or primer extension.

## RESULTS

**5.8S rRNA precursors can be detected by FISH in the cytoplasm.** To localize 3′-extended 5.8S precursors, fluorescent *in situ* hybridization (FISH) was performed using a 20-nt locked nucleic acid (LNA) probe (39). The ITS2-1 probe is complementary to the region traversing the 5.8S-ITS2 junction and hybridizes to all precursors of 5.8S, including 6S and 7S, but not to the mature rRNA. This probe gave a strong nucleolar signal, corresponding to nascent transcripts of 35S, 27S, and 7S pre-rRNAs. However, a weak signal throughout the cytoplasm was also observed in three different wild-type (WT) strains tested (Fig. 2A), indicating that the presence of cytoplasmic pre-5.8S species may be a general feature of yeast pre-rRNA processing.

To determine whether the cytoplasmic pre-rRNA species were being actively exported, FISH analyses were performed in a strain defective for ribosome subunit export. A single amino acid alteration ($T_{539}C$) in the gene encoding the exportin Crm1 renders it sensitive to inhibition by leptomycin B (LMB) (29). Following LMB treatment, the cytoplasmic signal in the *crm1*($T_{539}C$) strain was clearly decreased relative to the *CRM1* strain (Fig. 2B). This indicates that pre-60S particles contain-

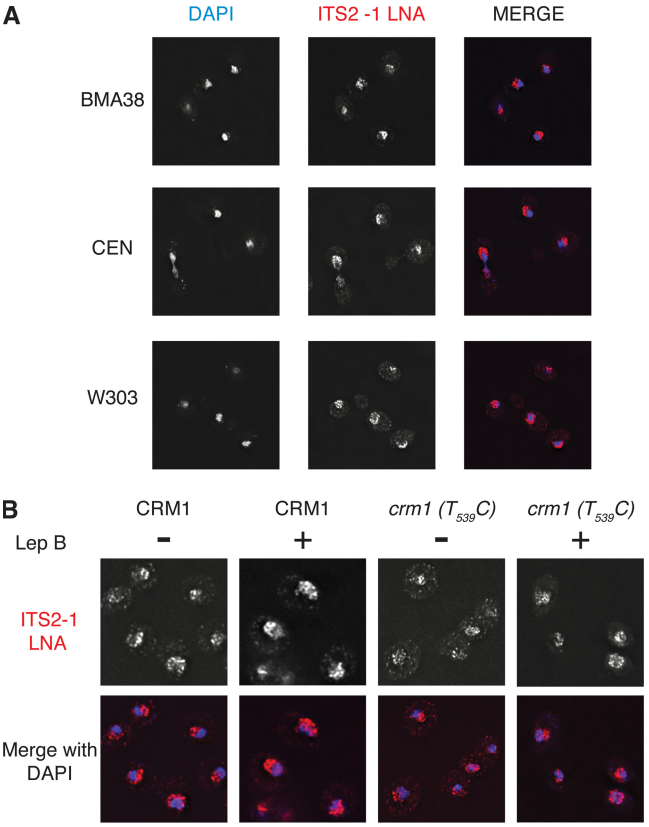



FIG. 2. A cytoplasmic signal corresponding to a 3′-extended 5.8S pre-rRNA can be detected. (A) Fluorescent *in situ* hybridization (FISH) was performed using the Cy3-labeled ITS2-1 LNA probe (red in merge panels) in various WT strains. Cells were fixed in paraformaldehyde and spheroplasted with Zymolyase. FISH was performed as described in Materials and Methods. The nucleoplasm was visualized by DAPI (blue in merge panels). (B) Wild-type (*CRM1*) and *crm1*($T_{539}C$) strains were grown at 25°C and, where indicated (+), treated with LMB (Lep B; 100 ng ml$^{-1}$) for 1 h. Cells were fixed in paraformaldehyde and spheroplasted. FISH was performed using the ITS2-1 LNA Cy3-labeled probes (red in merge panels). The nucleoplasm was stained with DAPI. These figures show a single optical section from a deconvolved stack. All images shown in panels A and B, respectively, were captured using identical exposure times. Following acquisition, data were processed in an identical fashion for each image.

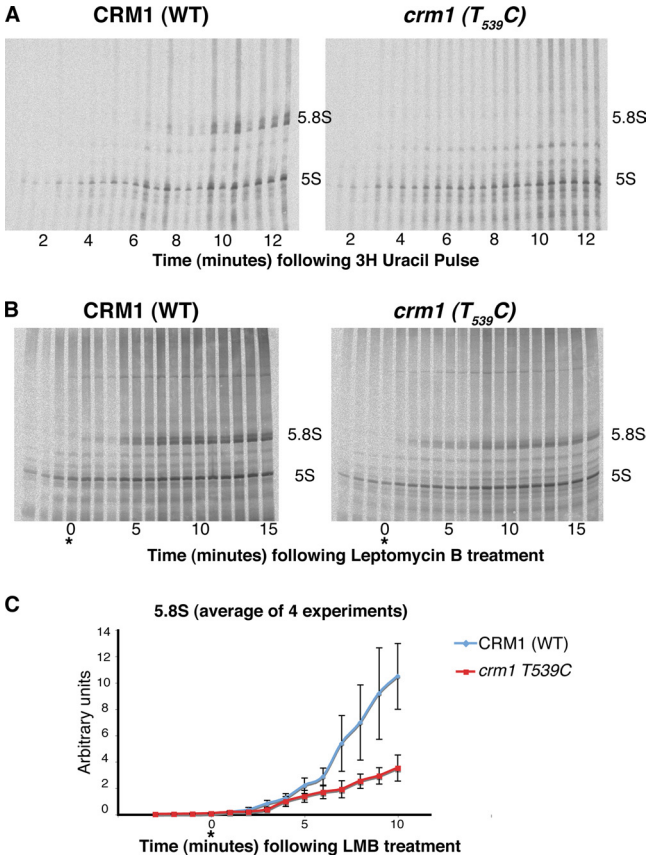



FIG. 3. 5.8S rRNA synthesis is rapidly inhibited when nuclear-cytoplasmic export is blocked. (A) Wild-type (*CRM1*) and *crm1*($T_{539}C$) strains were transformed with a p*URA* plasmid and grown in SD-Ura media. *CRM1* and *crm1*($T_{539}C$) strains were treated with LMB (100 ng/ml) for 15 min prior to labeling with [5,6-3H]uracil (GE) (1 mCi in 25 ml culture). One milliliter of cells was harvested every 30 s. Low-molecular-weight RNA was separated on an 8% polyacrylamide–8.3 M urea gel. (B) *CRM1* and *crm1*($T_{539}C$) strains were labeled with [5,6-$^3$H]uracil (GE) (1 mCi in 25 ml culture) for 5 min before treatment with LMB (100 ng/ml). Time of LMB addition is shown by *. One milliliter of cells was harvested at each time point indicated. Low-molecular-weight RNA was separated on an 8% polyacrylamide–8.3 M urea gel. (C) Experiments as shown in panel B were performed in quadruplicate, and tritiated $5.8S_{L+S}$ was quantified using a Fuji imager system with an FL-5100 screen. Lane background signal (tritium) was subtracted, and loading variations were accounted for by normalizing against the signal gained from a Northern hybridization using a $^{32}$P-labeled probe against 5S rRNA. To compare experiments, each data set was normalized to 5S $^3$H signal at the time point taken 1 min before LMB treatment. The averages of 4 experiments are shown. Error bars represent ±1 standard error.

ing 3′-extended precursors to 5.8S rRNA are actively exported to the cytoplasm.

**5.8S rRNA synthesis is rapidly inhibited in an export mutant.** If the exported pre-60S particles predominately contain 5.8S rRNA precursors, then blocking export should lead to a rapid inhibition of 5.8S synthesis. To assess the effect of inhibiting export on 5.8S rRNA synthesis, *in vivo* pulse-labeling with [$^3$H]uracil was performed in *CRM1* and *crm1*($T_{539}C$) strains. In initial experiments, cells were treated with LMB for 15 min prior to labeling with [$^3$H]uracil (Fig. 3A). Under these conditions, no mature 5.8S rRNA was synthesized in the *crm1*($T_{539}C$) mutant strain. In contrast, the independently transcribed 5S rRNA accumulated with identical kinetics in *CRM1* and *crm1*($T_{539}C$) strains. This indicates that treatment with LMB caused a rapid block in the synthesis of 5.8S rRNA.

To better define the kinetics of the inhibition of 5.8S synthesis, *in vivo* labeling was repeated with preincubation for 5 min with [$^3$H]uracil prior to treatment with LMB. Incorporation into 5.8S rRNA is seen during preincubation prior to drug treatment, but the rates of incorporation in the wild-type and mutant strains rapidly deviate following LMB addition (Fig. 3B and C). As early as 5 min after LMB addition, a reduction in 5.8S synthesis was evident, and by 7 min the difference in accumulation was significant.

The 3′-extended precursors to 5.8S rRNA, the 7S, 5.8S+30, and 6S pre-rRNAs, are not readily quantified by *in vivo* labeling. These were therefore analyzed by Northern hybridization (Fig. 4A) using probe 020, which hybridizes across the 5′ end of ITS2 (see Fig. 1A). In the *CRM1* wild-type (WT) strain, the ratio of 6S to 7S remains relatively constant, as would be expected for unperturbed steady-state RNAs. In contrast, the ratio was drastically affected in the LMB-sensitive strain, where the level of 6S fell with respect to 7S. A striking reduction of 6S levels could be seen following 6 min of LMB treatment, just prior to the marked decrease seen for 5.8S synthesis detected by pulse-labeling (Fig. 4B).

No accumulation of the 7S or 5.8S+30 pre-rRNAs was seen, suggesting that the decrease in levels of 6S pre-rRNA is not caused by inhibition of 7S-to-6S processing, but rather is due to 6S pre-rRNA being rapidly degraded when export is blocked. To test this prediction, we deleted the gene encoding Trf4, the poly(A) polymerase from the major TRAMP nuclear RNA surveillance complex (24, 43), in the *CRM1* and *crm1*($T_{539}C$) strains. In the *CRM1 trf4*Δ strain, there was little variation in the 6S/7S ratio (Fig. 4B), consistent with previous analyses that showed no major alteration in pre-rRNA levels in *trf4*Δ strains (19). In the LMB-sensitive *crm1*($T_{539}C$) *trf4*Δ strain, the 6S/7S ratio initially increased, following 5 min of LMB treatment, consistent with an inhibition of 6S processing, followed by a modest decrease. The 6S/7S ratio was clearly stabilized in the *crm1*($T_{539}C$), *trf4*Δ double mutant relative to the *crm1*($T_{539}C$) single mutant. This indicates that when export is blocked, 6S pre-rRNA is synthesized but is subject to degradation, for which Trf4 is partially responsible.

The rapid kinetics and specific inhibition of the 6S-to-5.8S synthesis suggest that blocking export is the direct cause of the defect, rather than a secondary effect. We conclude that pre-60S export is required for 3′ processing of 6S pre-rRNA to 5.8S rRNA.

**Shuttling and cytoplasmic 60S synthesis factors precipitate 6S pre-rRNA.** To determine which 5.8S precursors are associated with the exported pre-60S particles, immunoprecipitation was performed using three TAP-tagged bait proteins, Arx1, Nmd3, and Lsg1, which associate with different sets of late pre-60S particles (Fig. 5A). Copurifying RNAs were analyzed by Northern hybridization and primer extension. Arx1 associates with pre-60S particles in the nucleus and accompanies the pre-60S particles to the cytoplasm. As previously reported (30), Arx1 was strongly associated with 7S pre-rRNA. However, additional processing intermediates, including the 6S pre-rRNA, and the mature 5.8S were also enriched (Fig. 5B and

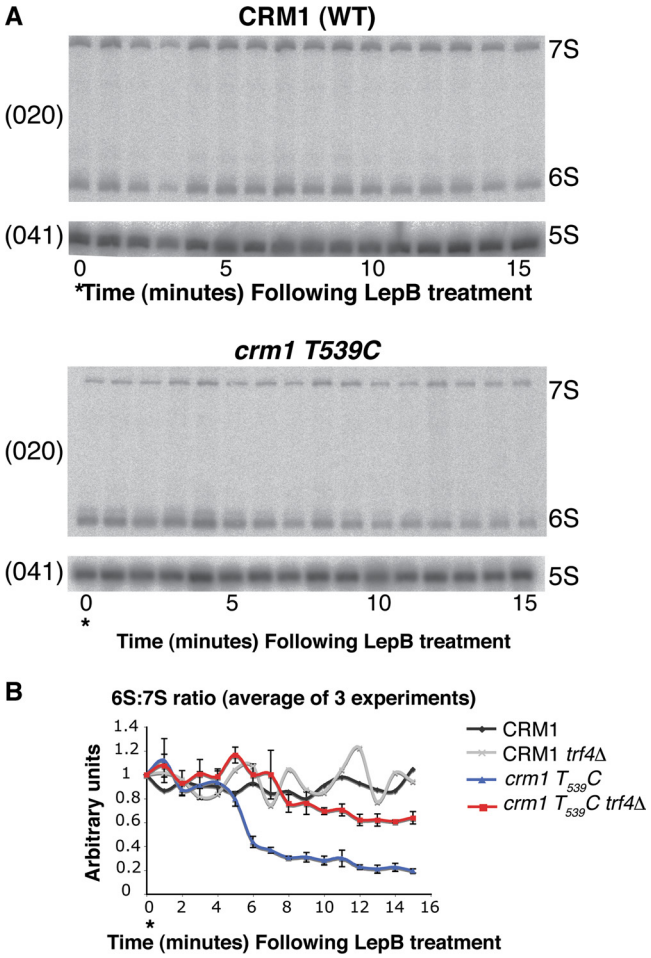



FIG. 4. 6S pre-rRNA levels decrease when nuclear-cytoplasmic export is blocked. (A) Wild-type (*CRM1*) and *crm1*($T_{539}C$) strains were treated with LMB (LepB; 100 ng/ml), at the 0-min time point (shown by ∗). Low-molecular-weight RNA was separated on an 8% polyacrylamide–8.3 M urea gel. Following transfer, membranes were probed with $^{32}$P-labeled oligonucleotide probes. $^{32}$P signal for 7S, 6S, and 5S was imaged using a Fuji imager system and quantified using AIDA software. (B) Quantification of the 6S/7S ratio from experiments performed in triplicate. The 6S/7S ratio was standardized by normalizing the first time point (0 min) to 1. Error bars represent ±1 standard error. For clarity, error bars have been excluded for the WT and the *trf4*Δ single mutant.

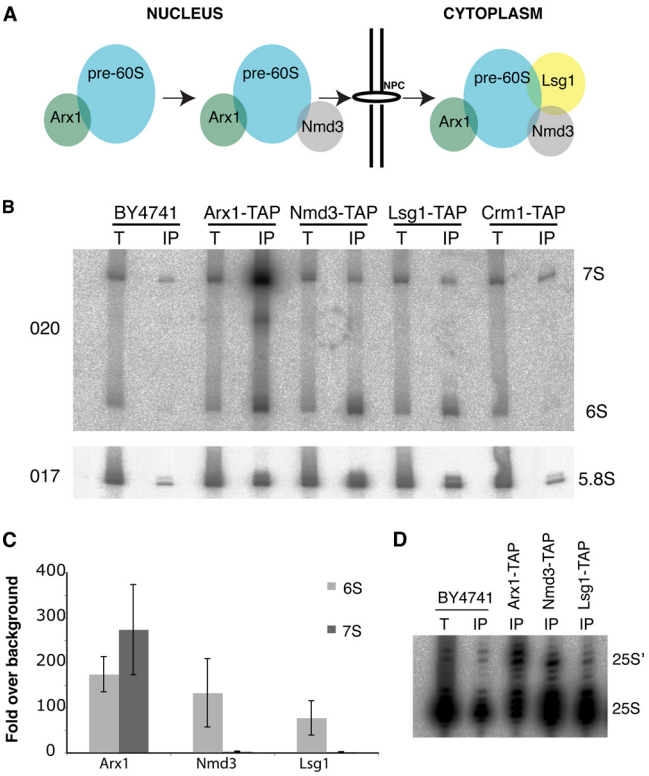



FIG. 5. The cytoplasmic GTPase Lsg1 precipitates 6S pre-rRNA. (A) Assembly of late pre-60S factors. Arx1 associates in the nucleus and shuttles with the pre-60S particles to the cytoplasm. Nmd3 is the export adapter protein that tethers the 60S preribosome to the export receptor Crm1/Xpo1. Once in the cytoplasm, the cytoplasmic GTPase Lsg1 releases Nmd3 from the 60S subunit. (B, C, and D) Northern and primer extension analysis of RNAs precipitated from the untagged strain (BY4741), Arx1-TAP, Nmd3-TAP, Lsg1-TAP, and Crm1-TAP. Cell lysates from the TAP-tagged strains were incubated with IgG-Sepharose, and the associated RNA was extracted from beads (IP). RNA from total cell extract was also prepared (T). Total RNA loaded corresponds to 1% of the amount used as input for immunoprecipitations. (B) To analyze low-molecular-weight species, RNA was separated on a denaturing 8% polyacrylamide–8.3 M urea gel. Following migration, RNAs were transferred to a nylon membrane and hybridized with the 5′-labeled oligonucleotide probes shown in parentheses on the left of the gel panels. (C) The experiment shown in panel B was performed in triplicate, and the data were quantified. The band intensity for precipitated 7S and 6S was quantified after subtraction of the lane background and expressed as a fraction of the corresponding signal in the total input lane to determine the efficiency of precipitation. The fold over background is the ratio between the efficiency of RNA coprecipitation seen with each protein and the background recovery of 7S/6S in the untagged BY4741 strain. (D) Primer extension analysis of the 5′ end of 25S, using oligonucleotide 007, where total RNA loaded corresponds to 1% of the amount used as input for immunoprecipitations. Reactions were analyzed on a denaturing 6% polyacrylamide–8.3 M urea gel, and images were captured using the Fuji imager system.

C). Nmd3 is the major export adapter for pre-60S particles and is proposed to be among the last factors that bind prior to export. The Nmd3 precipitation therefore characterizes an even later nucleoplasmic stage than Arx1. Consistent with this, Nmd3 did not enrich 7S pre-rRNA, compared to either the tagged or untagged control precipitations, but did enrich 6S pre-rRNA and 5.8S (Fig. 5B and C). This indicates that very late nucleoplasmic preribosomes that shuttle to the cytoplasm contain the 6S pre-rRNA. Finally, the cytoplasmic GTPase Lsg1 also showed enrichment of 6S and 5.8S. Lsg1 is essential for release of Nmd3 and for 60S subunit synthesis (16), and its association of with 6S pre-rRNA indicates that Lsg1 is present in the pre-60S particles that are exported to the cytoplasm. In contrast, purification of the exportin Crm1 yielded no coprecipitated pre-RNAs. During 60S export, Crm1 is associated with Gsp1 (yeast RAN) bound to GTP (reviewed in reference 22). On arrival in the cytoplasm, the RAN-GAP Rna1 stimulates hydrolysis of Gsp1-GTP to Gsp1-GDP, triggering pre-60S release. Following cell lysis, we expect that GTP hydrolysis by Gsp1 will be stimulated by Rna1, resulting in the dissociation of Crm1 from its preribosome cargo and preventing recovery of associated RNAs.

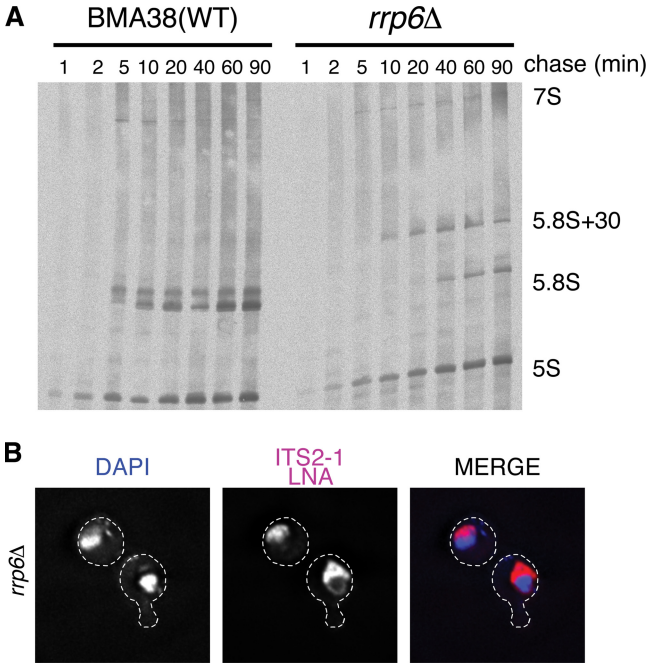



FIG. 6. Nuclear localization of 5.8S precursors in *rrp6*Δ mutants. (A) Pulse-labeling in wild-type and *rrp6*Δ strains. Cells were grown in SD-Ura media and pulse-labeled with [8-$^{3}$H]uracil for 2 min followed by a chase with a large excess of cold uracil for the times indicated. Low-molecular-weight RNA was separated on an 8% polyacrylamide–8.3 M urea gel. (B) FISH performed in an *rrp6*Δ strain. The LNA modified oligonucleotide ITS2-1 is shown as red in merge panels, and the nucleoplasm was visualized by DAPI (blue in merge panels). The cell periphery is shown by a dashed line.

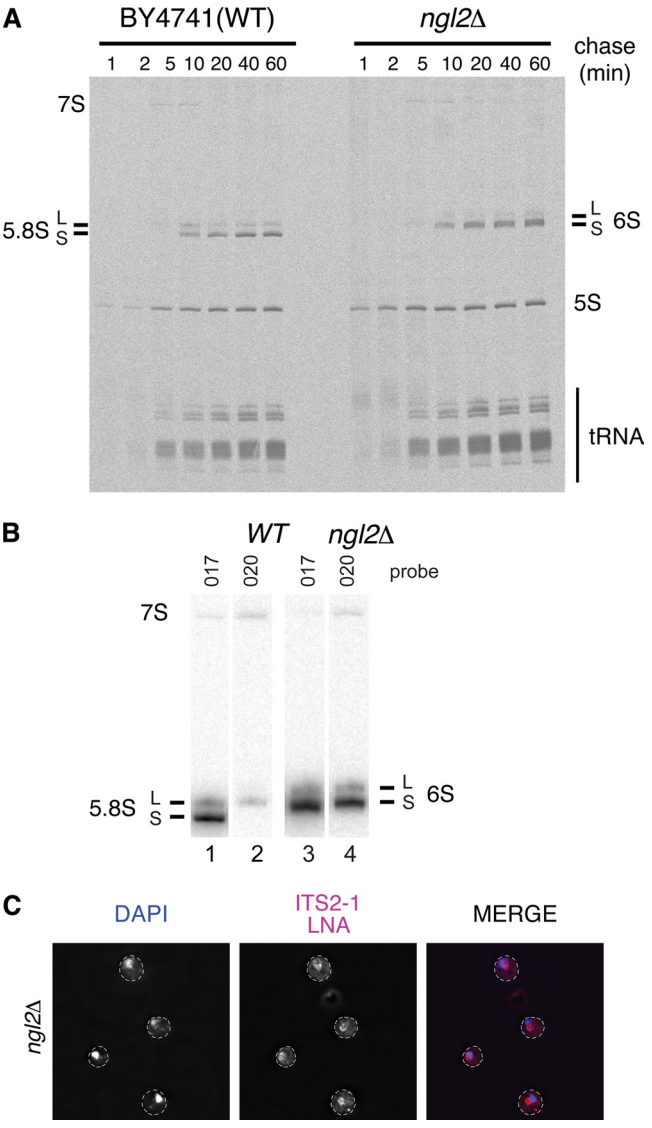


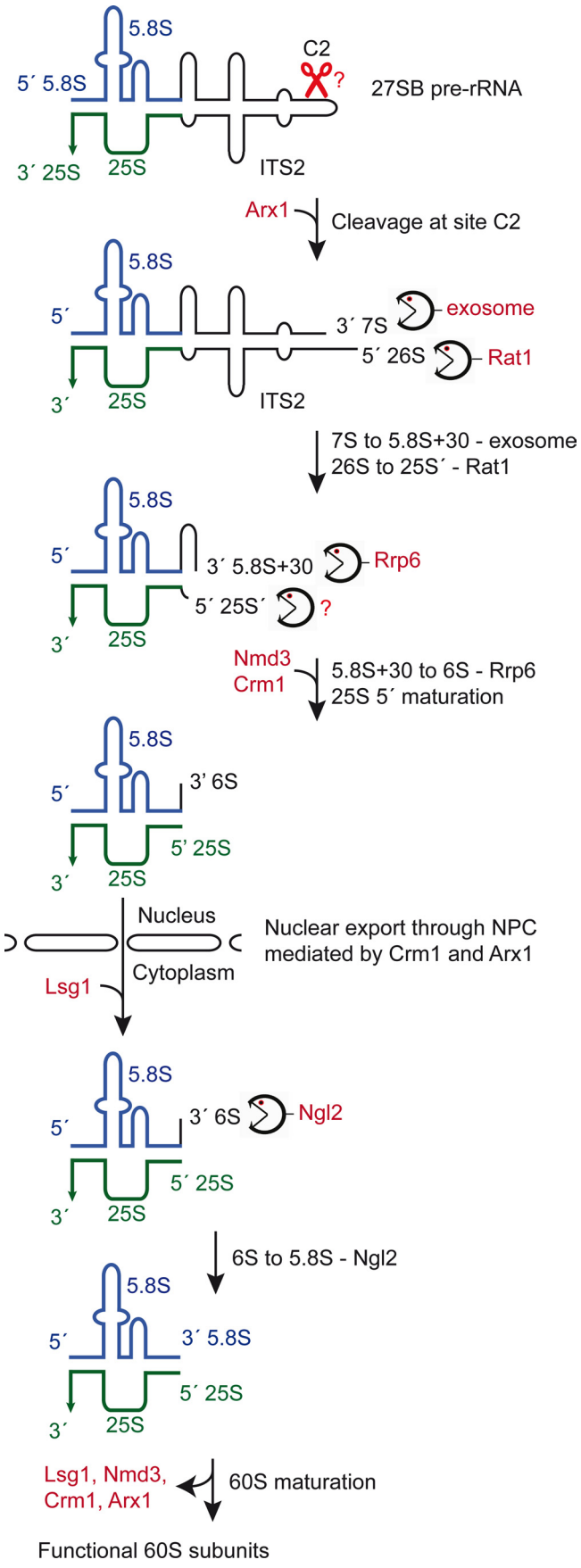

FIG. 7. Cytoplasmic localization of 5.8S precursors in *ngl2*Δ mutants. (A) Pulse-labeling in wild-type and *ngl2*Δ strains, performed as described for Fig. 6A. (B) Northern hybridization of the same filter (10-min time point lanes are shown) using probes hybridizing within 5.8S rRNA (017) or across the 5.8S-ITS boundary (020). The locations of the long (L) and short (S) forms of 6S and 5.8S are indicated. (C) FISH on *ngl2*Δ, performed as described for Fig. 6B.

The 25S rRNA is 5′ matured within late pre-60S particles, and this was analyzed by primer extension using RNA precipitated with the same bait proteins. Only Arx1-TAP precipitated the 5′-extended 25S′ species, which is extended by some 8 nucleotides at the 5′ end (Fig. 5D). This indicates that 5′ maturation of 25S rRNA takes place in late, nucleoplasmic pre-60S particles prior to export.

We conclude that the exported pre-60S particles contain mature 25S rRNA together with the 6S pre-rRNA.

**Localization of 5.8S precursors in processing mutants.** Previous analyses have revealed a multistep 3′ processing pathway for the 5.8S rRNA (see Fig. 7 below) (1, 8, 10, 44). The 7S pre-rRNA (5.8S 3′ extended by 150 nt) is generated by 3′ cleavage at site C2 by an unidentified endonuclease. The nuclear exosome then processes from 7S to the 5.8S+30 pre-rRNA (5.8S 3′ extended by ~30 nt) (1), followed by processing from 5.8S+30 to 6S (5.8S 3′ extended by ~6 nt) by the nuclear exonuclease Rrp6 (8). Finally, Ngl2 processes 6S to mature 5.8S (10). Ngl2 was reported to be cytoplasmic in genome-wide green fluorescent protein (GFP) localization studies (20, 23); however, we were unable to clearly localize Ngl2-GFP expressed at endogenous levels (data not shown). These data suggest that strains lacking Rrp6 should accumulate nuclear 5.8S+30 pre-rRNA, whereas strains lacking Ngl2 would accumulate 6S pre-rRNA in the cytoplasm.

Strains lacking Rrp6 or Ngl2 are viable (8, 10), and we therefore performed pulse-chase labeling in deletion mutants to confirm the defects in processing of newly synthesized pre-rRNA (Fig. 6A and 7A). In the *rrp6*Δ strain, an initial, complete block to mature 5.8S rRNA synthesis was seen with strong accumulation of 5.8S+30 (Fig. 6A). A low level of mature 5.8S rRNA was synthesized only at later time points, but there was a substantial delay between the appearance of labeled 5.8S+30 (10 min) and mature 5.8S (40 min). This long delay suggests that in the absence of Rrp6, no 5.8S+30 undergoes processing through the normal pathway and confirms that Rrp6 is indeed solely responsible for normal processing of 5.8S+30. However, alternative pathways can eventually mature some of the accumulated pre-rRNA. The localization of the accumulated pre-5.8S+30 RNA was assessed using the ITS2-1




FISH probe (Fig. 6B), which revealed very strong decoration of the nucleolus. Additionally, many *rrp6*Δ cells exhibited pre-5.8S signal at the nuclear periphery, presumably corresponding to pre-60S particles that contain 5.8S+30 and fail to be efficiently exported. The cytoplasmic signal was not clearly above the background in *rrp6*Δ, although the strength of the nucleolar signal made this difficult to image. In previous analyses, 5.8S+30 was detected in polysomes (8), indicating that some leakage does take place.

In the *ngl2*Δ strains, the 6S pre-rRNA species accumulated and no mature 5.8S rRNA was synthesized (Fig. 7A), confirming that Ngl2 is solely responsible for normal processing of 6S pre-rRNA. To confirm the identities of the RNA species observed by pulse-labeling, the same filter was hybridized with $^{32}$P-labeled probes directed against 3′-extended 5.8S (probe 020) or the mature 5.8S rRNA (probe 017) (Fig, 7B; the lanes corresponding to the 10-min pulse-chase samples are shown). FISH analyses were performed in the *ngl2*Δ strain to localize the 6S pre-rRNA (Fig. 7C), which showed a strong signal throughout the cytoplasm.

We conclude that the 5.8S+30 pre-rRNA is predominately nuclear restricted, whereas 6S pre-rRNA accumulates in the cytoplasm, consistent with the immunoprecipitation data (Fig. 5).

## DISCUSSION

The data we present here provides the first direct evidence in yeast of a cytoplasmic phase of 5.8S rRNA maturation. Based on the data presented, we propose a revised model for the synthesis of 5.8S rRNA (Fig. 8). This proposes that nuclear pre-60S particles that contain 6S pre-rRNA are competent for binding the export adapter Nmd3 and exportin Crm1 and are exported to the cytoplasm. Once in the cytoplasm, the GTPase Lsg1 binds the pre-60S and the putative nuclease Ngl2 completes the 3′ maturation of 5.8S rRNA. Finally, Nmd3 is displaced by Lsg1 and recycled to the nucleus.

These findings may help answer the question of how and when ribosome subunits attain export competence and how immature subunits are excluded from the, essentially irreversible, step of nuclear export. The finding that 6S pre-rRNA was the only precursor species coprecipitated with Nmd3 (Fig. 5A and B) indicates that removal of ~20 nt of RNA from 5.8S+30

FIG. 8. Model for the final steps in 25S and 5.8S processing. Cleavage of the 27SB pre-rRNA at the $C_2$ site, by an as yet unidentified enzyme, generates the 7S and 26S pre-rRNA species. Following this cleavage, the processing and export factor Arx1 associates with the preribosomal particle. The 7S pre-rRNA is trimmed to a 5.8S+30 species by the 3′→5′ exonuclease activity of Rrp44, a component of the nuclear exosome. The 26S species is processed by the 5′→3′ exonuclease Rat1 to the 25S′ pre-rRNA, which is extended at the 5′ end by some 8 nucleotides compared to the mature 25S. This species is trimmed back to the mature form, probably through the activity of Rat1. The 5.8S+30 form is processed to 6S pre-rRNA by the nuclear exonuclease Rrp6. The 25S- and 6S-containing preribosomes are competent for export to the cytoplasm and associate with export adapter molecule Nmd3, which recruits the export receptor Crm1. Following export through the nuclear pore complexes (NPC), the recycling factor Lsg1 associates with the preribosome. 6S pre-rRNA processing to the mature 5.8S is completed by Ngl2. Finally, the remaining nonribosomal factors dissociate from the subunit, generating functional 60S subunits.

to 6S is coupled to the acquisition of export competence. The simplest hypothesis might be that this region binds one or more protein factors that are lost on processing. These factors might mediate nuclear retention by preventing binding of Nmd3, either directly or by imposing a particular preribosome structure. Experiments to characterize the proteins bound to this region of the pre-rRNA are under way.

The TRAMP4 complex, containing Trf4, is involved in the turnover of the nuclear retained 6S pre-rRNA when export is blocked. The TRAMP complexes act through addition of short poly(A) tails to the RNAs to be degraded and target RNAs for exosome-mediated degradation. We predict that when export is blocked the nuclear retained pre-rRNAs are subject to oligoadenylation and subsequent degradation. The incomplete stabilization observed in the *crm1*($T_{539}C$) *trf4*Δ strain is likely due to the activity of additional surveillance factors. Functional redundancy appears to be a general property of the nuclear RNA surveillance system (reviewed in reference 18). In addition to Ngl2, mutations in a surprising number of other 3′ exonucleases have been shown to accumulate 6S pre-rRNA, including members of the RNase D family of exonucleases Rex1, Rex2, and Rex3 (44) and core components of the exosome (1). We speculate that this 6S accumulation reflects reduced surveillance of late, nuclear preribosomal particles. Export of preribosomes to the cytoplasm is a key, irreversible step in ribosome synthesis, and other analyses suggest that surveillance of RNA-protein complexes is less active in the cytoplasm than in the nucleus (reviewed in reference 18). Preribosome surveillance may therefore be particularly stringent for the late, 6S-containing pre-60S particles immediately prior to export.

It is clear that more maturation of the pre-60S particle occurs in the cytoplasm than was anticipated. In recent years, a class of purely cytoplasmic ribosome synthesis factors have been identified. These include Lsg1, Drg1, and Rei1 (21, 25, 32), which are involved in the recycling of shuttling ribosome synthesis factors and reveal the elaborate cytoplasmic rearrangements that pre-60S particles undergo. The finding that final maturation of the 5.8S rRNA occurs in the cytoplasm further underlines the complexity of the cytoplasmic maturation phase.

The 3′-end maturation of 5.8S rRNA may also be a cytoplasmic event in metazoans. In *C. elegans* and mouse cells, the final step of 5.8S processing requires the RNase T-like exonuclease Eri1, which is also a negative regulator of RNA interference (2, 12). In *C. elegans*, Eri1 is cytoplasmic, suggesting that 5.8S maturation is likewise cytoplasmic. No clear homologue of Eri1 exists in *S. cerevisiae*, but Ngl2, which performs the equivalent role, is found in the cytoplasm. This final step in 5.8S processing is apparently nonessential for production of functional ribosomes, as both yeast *ngl2*Δ strains and *eri-1* null worms are viable (10, 12). It is possible that formation of the 6S pre-rRNA principally acts as a signal for export competence, while its maturation to 5.8S rRNA signals the completion of export.

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